DEVELOPMENT AND VALIDATION OF IMMUNODIAGNOSTIC METHODS FOR QUANTITATIVE ESTIMATION OF RAT C-PEPTIDE

Synopsis sunmitted
in partial fulfillment of Degree of
Doctor of Philosophy
in Applied Zoology

By
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Diabetes mellitus (DM) is a predominantly concerned health challenge and becoming a significant cause of death worldwide, affecting a large population both in developing and developed countries (Eluehike and Onoagbe 2020). According to the World Health Organization (WHO), the prevalence of DM was increasing and was the ninth leading cause of mortality in 2019. Over 1.56 million deaths were reported directly caused by diabetes (WHO 2021). DM is a metabolic disorder that occurs because of deficient insulin production or results from ineffective insulin use from the body (Adienbo and Hart 2021). It causes blindness, kidney failure, heart attacks, stroke and lower limb amputation (WHO 2021). There are two major types of DM: Type I and Type II. Proinsulin is the biosynthetic precursor of insulin (Steiner *et al*., 1969), which is cleaved into insulin and C-peptide. Both are released from the pancreatic β-cell into the circulation in equimolar amounts. The metabolic clearance rate of C-peptide is the slowest among the three polypeptides proinsulin, insulin and C-peptide. Hence, C-peptide is considered a unique diagnostic tool in diabetology as it is an independent marker for endogenous insulin secretion in the body (Horwitz *et al*., 1975). The measurement of C-peptide in the blood reflects the insulin concentration in the bloodstream and also evaluates the pancreatic β-cell function (Horwitz *et al*., 1997).

In the rat, preproinsulin is synthesized in the isoforms I and II, cleaved into insulin I and II and C-peptide I and II. Rat C-peptides I and II are single-chain peptides composed of 31 amino acids with a homology of 93.5 % (Hytest Technotes 2011). C-peptide demonstrates considerable interspecies variability. Most of the species have only one form of proinsulin. Nevertheless, rats and mice produce two proinsulin isoforms-I and II, which differ by two (rat) and three (mouse) amino acid residues of the C-peptide part of proinsulin. (Horwitz *et al*., 1975). The amino acid sequence of the rat C-peptide isoforms is elaborated below:

NH3+- Glu – Val – Glu – Asp – Pro – Gln – Val – Pro – Gln – Leu – Glu – Leu – Gly – Gly –Gly – Pro – Glu – Ala - Gly – Asp – Leu – Gln –Thr - Leu- Ala – Leu - Glu – Val – Ala – Arg – Gln - CO2- = RAT I

NH3+ - Glu - Val – Glu – Asp – Pro - Gln - Val - Ala - Gln - Leu -Glu - Leu - Gly - Gly - Gly – Pro - Gly - Ala - Gly – Asp - Leu - Gln -Thr - Leu- Ala - Leu - Glu – Val – Ala – Arg –Gln - CO2- = RAT II

Amino acid sequences of rat C-peptide I and II. (Tager and Steiner 1972)

Various immunoassay methods are presently employed to measure C-peptide in human plasma (Liu *et al*., 2014; Thomas *et al*., 2020; Owusu *et al*., 2021). However, the wide species variations in primary structures preclude using heterologous assays for C-peptide measurements in other species (Tager and Steiner 1972). The rat is a widely used model for studies of diabetes (Peterson *et al*., 1990 and Wei *et al*., 2003), islet transplantation (Korsgren *et al*., 2008), hypoglycemia and insulinoma (Sugimoto *et al*., 1996 and Striano *et al*., 2003). Quantification of C-peptide in rat serum is highly promotable, as its studies are directly related to the diagnosis of carbohydrate metabolism abnormalities, pancreatic performance analysis, monitoring of hypoglycemia and diabetes-related illness in the rat model. The reliable measurement of rat C-peptide is not possible with human C-peptide assays, so an assay design for rat C-peptide quantification is essential.

Immunoradiometric assay (IRMA) is a method for directly estimating analytes in biological fluids based on radio isotopically labelled specific antibodies. The principle of IRMA is relative to the excess use of radio-labelled antibodies with varying concentrations of the analyte. Excess antibody addition forces the faster equilibrium reactions by shortening the incubation time. IRMA is, therefore, more rapid than RIA. Calibration curves are of exponential type. As the analyte concentration increases, more binding occurs, giving more signals within the constraint time and reagents. In the earliest IRMA, polyclonal antibodies were labelled and used excessively with the solid phase antigen. The supernatant labelled antigen-antibody complex was aspirated and counted after the centrifugation. This method failed due to the technical difficulty in preparing labelled antibodies using polyclonal antibodies. With many changes, one more variant of IRMA was developed, two-site immunoradiometric assays. This method used one monoclonal antibody as solid-phase support, and another was radio-labelled (tracer). Solid-phase antibodies were primarily incubated with the analyte and washed to remove the unreacted analytes. Secondary incubation was with a radio-labelled antibody which reacted with alternative antibody binding sites of the analyte-solid phase complex. The repeated washing step removes the entire unreacted labelled antibody from the system. The addition of antibodies in excess has been reported to reduce the time required for reactants to reach equilibrium, remove the need for precise pipetting of antibodies and increase the "operating range" and sensitivity (Ekins *et al*., 1976). The first two-site immunoradiometric assay for rat proinsulin was developed by Yue *et al*., (1979) using rabbit antibody and reported a sensitivity of 0.006 pmol/mL.

Though many radio-iodination methods are available, the radio-iodination of protein by chloramine-T (CAT) method (Greenwood *et al*., 1963) is preferred for its simple steps involved, low amount of reagent requirement and quick reaction with a good radio-iodination yield of around 60-80 %. CAT can oxidize radioactive iodine salts and release radioactive elemental iodine, which gets conjugated with the tyrosine or histidine residue of the protein (Tashtoush *et al*., 2001). The main advantage of this method is that it is widely applicable to various proteins, giving high specific activity. The potent oxidizing agent CAT and reducing agent, sodium metabisulfite method, would yield a damaged labelled protein with less immunoreactivity. However, the effect can be minimized by using a controlled amount of oxidizing and reducing agents and by designing the other reaction conditions (Wood *et al*., 1981).

In IRMA, separation of the antigen-antibody complex from the unreacted antigens is an essential step to be carried out. Still, it is prone to error, or it would be a time-consuming step. And also, the choice of separation step will interfere with the sensitivity and reproducibility of the assay (Vrinda *et al*., 2003). Adopting various solid-phase separation methods would lead to a simpler and more reliable IRMA method. Plastic tubes are a very convenient separation method, but they are expensive, need good skills to handle and are also difficult to control at all the stages of manufacture (Jyotsna *et al*., 1998). So, the simple and inexpensive separation methods using magnetic particles were followed in later years.

Enzyme-Linked Immunosorbent Assay (ELISA) is one of the most widely used biochemical methods in laboratory analysis and diagnostics. Analytes such as peptides, proteins, antibodies and hormones can be detected selectively and quantified in low concentrations, among many other substances. In the basic principle of ELISA, enzyme molecules are conjugated to secondary antibodies, which bind to the primary antigen-antibody complex. When the appropriate substrate is added, the enzyme catalyzes the production of a coloured end-product, which can be visualized and quantitated. The enzymes most often used are alkaline phosphatase (AP) and horseradish peroxidase (HRP) (Koivunen and Krogsrud 2006). There are four types of ELISA, indirect ELISA, competitive ELISA, sandwich ELISA and direct ELISA.

In indirect ELISA, a limited amount of serum containing primary antibodies is added to the microtiter plate coated with varying analyte concentrations. The free primary antibody is washed out and treated with an enzyme-conjugated secondary antibody that binds to the primary antibody. Unbound secondary antibody is washed away, and a specific

substrate is to be added. The enzyme hydrolyzes the substrate and forms a coloured product and it is read at 450nm by the ELISA reader. The amount of colour developed is directly proportional to the concentration of analyte present.

The competitive ELISA procedure includes preparing a coated microtiter plate with a certain amount of analyte. The antigen-antibody mixture is added to the well coated with the antigen. The availability of free antibodies for coated antigens depends on the concentration of the antigen added to the mix. After the wash, an enzyme-conjugated secondary antibody is added and incubated. The addition of a specific substrate develops colour. Absorbance is inversely proportional to the concentration of the sample in the sample; the higher the analyte concentration gives the less absorbance.

The research of anti-diabetic drug discoveries needs a quantitative method to estimate C-peptide in rat serum. The aim of the work is to develop a cost-effective indigenous method that can be applied in any laboratory. Hence, the following objectives were set to validate IRMA and ELISA for rat C-peptide to provide a high degree assay with adequate sensitivity and specificity, which are necessary to quantify the C-peptide in rat serum in diabetic rat models.

1. Optimization of radio-iodination procedure for rat C-peptide and anti-rat C-peptide

with 125I radioisotope.

1. Development and validation of IRMA for rat C-peptide.
2. Development and validation of indirect and competitive ELISA for rat C-peptide.
3. Validation of the developed IRMA and ELISA methods with a commercially

available kit.

The prescribed thesis comprises six chapters, namely introduction, review of literature, materials and methods, results, discussion and summary.

Chapter 1 involves the background of diabetes and rat C-peptide, the importance of C-peptide in the field of drug testing for diabetes and the aim of the present study.

Chapter 2 comprises brief information from the literature on the structure and biosynthesis of rat C-peptide and its function in a biological system. The novel application of magnetic particles as a separation system in the IRMA is explained. The preparation of detector antibodies for IRMA by radio-iodination procedures like chloramine-T,

electrolytic, iodogen, and enzymatic peroxidase methods are outlined. It is followed by a brief note on the production of polyclonal antibodies and its application in ELISA. The review is followed by a write-up on the history of the application of polyclonal and monoclonal antibodies, assay principles and basic procedure of immunodiagnostic methods such as radioimmunoassay, immunoradiometric assay, indirect ELISA and competitive ELISA in the quantification of C-peptide in various species. The complications in the rat serum collection and storage are also discussed. In the end, the scope of the study and objectives selected to justify the proposed work is mentioned.

Chapter 3 describes the methodology followed to optimize IRMA, indirect ELISA and competitive ELISA. Magnetic particles used as a separation system were characterized by scanning electron microscopy, Fourier Transform Infrared Spectroscopy, X-ray Diffractometer and magnetic resonance spectrophotometry. IRMA method involves a reaction between detector antibody, capture antibody and rat C-peptide standards. The detector antibody was prepared by radio iodinating a monoclonal antibody with 125I using the chloramine-T oxidation method (Greenwood and Hunter 1963). The CDI activation method coupled the capture antibody to the magnetic particle (Kadwad *et al*., 1996). The detector antibody was prepared as follows: rat C-peptide monoclonal antibody was treated with chloramine-T and 125I for 60 sec, and the reaction was stopped by adding a reducing agent. The prepared reaction mixture was purified using a pre-equilibrated Sephadex G-75 column and fractions were collected. Radio-iodination yield of the reaction mixture and radiochemical purity of purified fractions were determined using paper electrophoresis. Detector antibody was dispensed into 1 mL and stored for further use. A matched pair was found by incubating a mixture of 100 µL of capture antibody (SM1 or SM2) and detector antibody (M1* or M2*) with the rat C-peptide in concentrations of 0, 0.625 and 20 ng/mL. After finding a suitable tracer and a matched pair, the IRMA assay was set up by adding 100 µL each of detector antibody (M1*), capture antibody (SM2) and three standards (0, 0.625 and 20 ng/mL), incubated for 3 hours on a shaker and separated by washing two times with 2 mL of assay buffer and keeping on the magnetic rack for 20 minutes. The rat C-peptide standards were prepared between 0 to 20 ng/mL in 0.05 M phosphate buffer, pH=7.4. The IRMA assay was optimized by taking the parameters like sample volume, capture antibody concentration and its titer, tracer counts, incubation time, washing step, and washing buffer volume to achieve a low non-specific binding and maximum binding.

Further quality control parameters such as recovery, parallelism and dilution linearity were tested to assess the assay. A typical standard curve for rat C-peptide IRMA was plotted using a log-log graph by taking rat C-peptide concentration in ng/mL on the x-axis and % B/T on the y-axis. The C-peptide concentration of around 30 rat serum samples was quantified using the optimized assay.

In the following section, the developed indirect and competitive ELISA technique for quantification of C-peptide in rat serum is discussed. To produce polyclonal antibodies against rat C-peptide, BSA was conjugated with the rat C-peptide by Mannich reaction in three ratios to improve immune response in the rabbit system. One of the ratios was selected to inject rabbits for the polyclonal antibody production. Institutional Animal Ethics Committee (IAEC), SCP/IAEC/F150/P167/2019, dated 27.12.2019, provided approval for the work. Rabbits were divided into three groups. Each group had two rabbits; one group was designated as control, and the other two as experimental. The rabbits in experimental groups were injected intramuscularly with a mixture of rat C-peptide- BSA conjugate and Freund's complete adjuvant after ten days of pre-bleed. With the gap of 15 days, the booster of rat C-peptide- BSA conjugate and Freund's incomplete adjuvant was given, and 10 mL of blood was collected after ten days of each booster. The serum was tested for the presence of polyclonal antibodies against rat C-peptide by indirect ELISA. ELISA microplate was coated with 6 ng/mL of rat C-peptide in 0.05 M phosphate buffer, pH=7.4 at 37^{o} C for 5 hours. The coated plate was blocked with 10 % skim milk for 2 hours and treated with 100 µL of serum containing polyclonal antibody. The plate was incubated overnight, and added 100 µL of 1:20000 diluted secondary antibodies. The plate was washed with PBST five times and treated with the substrate for 10 min and the reaction was stopped with 2 M sulphuric acid. Reading was taken at 450 nm. Soon after confirming the presence of polyclonal antibodies against rat C-peptide in the serum, it was purified with an IgG purification kit and titer was detected.

The indirect assay was performed by coating rat C-peptide standards 0-10 ng/mL to ELISA plates, blocked and allowed to react with primary antibody followed by secondary antibody, substrate and stop solution. The steps of indirect ELISA were optimized by considering the parameters like rat C-peptide concentration for standards, the concentration of blocking reagent, incubation time of blocking reagent, primary antibody, secondary antibody, the volume of sample, substrate and stopping solution and titer of primary and secondary antibody. Quality controls parameters like recovery, linearity, intra-assay and inter-assay variations were assessed.

Similarly, a competitive assay was optimized by coating the plates with rat C-peptide at 4º C or 37º C with varying concentrations. Further reactions with rat C-peptide standard, primary antibody and secondary antibody were done at 37º C. The optimizing parameters like concentrations of rat C-peptide standards, the volume of standards and primary antibody, titer of primary antibody, washing buffer and blocking reagent were determined along with the assessment of quality control parameters.

The developed Indirect and competitive ELISA was validated with the commercially available rat C-peptide ELISA kit with 30 rat serum samples. The IRMA and ELISA methods were compared using the rat C-peptide concentrations obtained in the serum sample.

Statistical analysis and illustrations were drawn using Microsoft excel 2019, Origin 9.0 software, and Inkscape 1.1 software.

Chapter 4 deals with the results of the work, which are presented under the following headings – (i) Characterization of magnetic particles (ii) Development of rat C-peptide IRMA (iii) Preparation of rat C-peptide-BSA conjugate (iv) Production of polyclonal antibody against rat C-peptide (v) Development of rat C-peptide indirect ELISA (vi) Development of rat C-peptide competitive ELISA and (vii) Correlation studies of developed methods with a commercial kit.

The presently designed research is the first report on development of a magnetizable cellulose particle-based rat C-peptide IRMA method. Rat C-peptide-BSA conjugation preparation by Mannich reaction, production of rat C-peptide polyclonal antibodies in rabbit system and development of an indigenous ELISA method are the new methods to benefit the researchers. Results on quantification of rat C-peptide by immunodiagnostic methods are discussed in Chapter 5 to focus on (i) Selection of separation system and radio-iodination method, (ii) Characterization of produced polyclonal antibodies, (iii) Assessment of assay quality parameters and (iv) Comparison of developed methods.

Salient features of the results and their significance are summarized in Chapter 6, the abstract of which is as follows:

Development of rat C-peptide IRMA:

1. Magnetized cellulose particles were characterized using scanning electron microscopy, Fourier Transform Infrared Spectroscopy spectra, X-ray

Diffractometer analysis and M-H curve. The mean size of the particles was found to be 2.5±0.5 µm, the minimum and maximum size observed was 0.3 and 2.8 µm, respectively. Several functional groups were detected from FTIR spectra, the peaks observed at 559.35 and 455.20 cm-1 correspond to the magnetite state vibration of the Fe-O bond. The peaks 3327.20, 2889.36, 1618.27, 1344.38 and 1004.91 cm-1are the characteristic peaks of cellulose in the solution. The peak 3327.20 cm-1represents – OH stretching observed due to the presence of deionized water. Other peaks 2889.36, 1618.27, 1344.38 and 1004.91 cm-1 symbolize the -CH stretching, -OH bending, -CH2 wagging, and C-OH stretching in the plane at C-6, respectively, with low intensities as iron particles are immobilized on the surface of cellulose. Certain functional groups of magnetic particles like aldehyde (–CHO), hydroxyl (–OH) and amine (–NH2) are mainly involved in the coupling process with the antibody. The X-ray diffractogram of magnetizable cellulose particles has mainly five 2θ degree reflections at 30.02°, 35.44°, 43.10°, 57.06° and 62.46°, with the crystalline structure ranging from 1.48 to 3.96 %. The shape of the peak was observed to be wider than the regular Fe3O4 XRD pattern, which indicates the incorporation of cellulose molecules into the magnetic particles. The magnetometry of the magnetized cellulose particles synthesized at room temperature measured was 60,000 to -60,000. A magnetic hysteresis loop can be noticed in the curve, demonstrating the superparamagnetic property of the particle with 0.14 emu/g magnetic saturation.

1. After comparing monoclonal antibody M1 tracer to rat C-peptide tracer and monoclonal antibody M2 tracer, the monoclonal antibody M1 tracer was chosen as a tracer or detector antibody. The radio-iodide activity of 0.6 mCi was found suitable for radio-iodinating M1 to produce an M1* tracer. M1* had a radio-iodination yield of 75.6 %, radiochemical purity of >90 % and was stable for six weeks.

1. The M1*-SM2 combination outperformed M2*-SM1 in the matched pair compatibility test. The non-specific binding and maximum binding achieved for M1*-SM2 were 0.7 and 24.5 %, respectively. So M1*-SM2 combination was selected as a suitable matched pair (detector and capture antibody pair) for IRMA.

1. Four different serums, such as horse serum, rat serum, bovine serum and human serum in charcoal treated and untreated forms, were used to select the suitable matrix for rat C-peptide standard preparation. Among these, horse serum gave the minimum NSB of 0.32 %. Hence, horse serum matrix was chosen for rat C-peptide standard preparation. The solid phase concentration of 20 g/per tube with a titer of 1:4 was utilized in the IRMA assay. Along with this sample volume of 100 µL, overnight incubation and tracer counts of 70000 cpm were well suited for the developed IRMA.

1. The following steps were involved in an optimized IRMA assay.
    1. The RIA tubes were designated by the letters T, A, B, C, D, E, C1, C2, R1, and R2.
    2. Tubes were filled with 100 µL of 1:4 diluted solidphase (SM2), 100 µL of 1:5 diluted tracer (M1*) and 100 µL of rat C-peptide standards 0-20 ng/mL, C1, C2, R1 (50 µL of C1 + 50 µL of 2.4 ng/mL rat C-peptide standard) and R2 (50 µL of C2 + 50 µL of 5 ng/mL rat C-peptide standard)
    3. Tubes were vortexed thoroughly and covered with aluminium foil before placing the rack on the shaker overnight.
    4. The following day, 2 mL of assay buffer (0.05 M phosphate buffer, pH=7.4 + 0.2 % BSA + 0.1 % tween 20) was added to the magnetic rack and left for 20 minutes. The supernatant was discarded and the process was repeated.
    5. Reading was taken on a multi-well gamma counter for a minute and recorded as counts per minute.
    6. A graph was constructed on a log-log graph and obtained a typical standard curve for rat C-peptide IRMA using the rat C-peptide concentrations of 0, 0.625, 1.25, 2.5, 5 and 20 ng/mL.

1. For the IRMA assay, two control values were established, ranging from 0.44 to 0.56 (control 1) and 8 to 12.8 (control 2), respectively and were expressed as 0.50±0.06 and 10.4±2.4 ng/mL for control 1 and control 2. Sample recovery ranged from 96 to 118 % in assay quality control parameter studies. The intra-assay variation was 3.8 to 7.1 %, whereas the inter-assay observed was 7.8 to 14

% for sample 1 and sample 2, respectively. The dilution linearity and parallelism ranged between 88-104 % and 92 -120 %, respectively.

1. Valuation of IRMA reagent's stability was also done. Rat C-peptide standards prepared in treated horse serum with 0.5 % trehalose were the most stable matrix. Furthermore, the addition of 0.1 % sodium azide to the rat C-peptide standards and solid-phase increased the quality of the reagents. The reagents' stability ranged from weeks to years. The tracer remained steady for 6 weeks, whereas the rat C-peptide standards were stable for four months and the solid phase was stable for over two years. As a result, the assay reagents can be stored at 4 °C for two months. The sensitivity of 0.24 ng/mL was achieved for the developed rat C-peptide IRMA. The concentration of rat C-peptide in 30 rat serum samples ranged from 0.24 to 0.612 ng/mL. Rat C-peptide concentration obtained from developed IRMA was compared with rat C-peptide concentration derived from commercially available rat ELISA kits.
2. The conjugation ratios of 1:20, 1:40 and 1:80 were prepared for rat C-peptide-BSA conjugates. The 1:40 ratio had a maximum absorbance of 0.718 and a band at ~69 kDa on the SDS-PAGE gel, indicating that the conjugation was effective. The same ratio was used to immunize the rabbits to produce polyclonal antibodies against rat C-peptide. Polyclonal antibodies were produced within three months of immunization and validated by indirect ELISA. Rabbit serum containing polyclonal antibodies against rat C-peptide was purified using an IgG purification kit. Polyclonal antibodies were detected in the third fraction, characterized by reading the fractions at 280 nm using UV-Visible spectrophotometry. Two bands were identified on SDS-PAGE gel at ~50 kDa and ~38 kDa.
3. Dot ELISA and western blot were used to determine the specificity of the produced polyclonal antibody. The minimal concentration of rat C-peptide produced colour on nitrocellulose membrane was 1.55 ng/mL (spotted 3 µL). When the nitrocellulose membrane was incubated with the primary antibody in dilution 1:50 and secondary antibody in dilution 1:1000, the band was detected at ~69 kDa in western blot. The working dilution of polyclonal antibodies was determined using 1:10, 1:100, 1:500, 1:1000, 1:2000, 1:4000, 1:8000, 1:16000, and 1:32000 dilutions of pure serum. In indirect ELISA, the polyclonal antibody dilution of 1:1000 gave the best results, and this was chosen as the dilution for the subsequent experiment.

4. The preparation of coated plates using rat C-peptide standards is the first step in the indirect ELISA that has been optimized. The standards 0, 0.625, 1.25, 2.5, 5 and 10 ng/mL were prepared in 0.05 M phosphate buffer, pH=7.4. The high binding ELISA plate wells were coated with rat C-peptide standards. The coating process was optimized at 4º C for a day or 37° C for 3 hours.
5. Coated plates were blocked using a blocking reagent of 1 % BSA and 5 % skim milk powder. Although both blocking reagents blocked the empty spaces in the wells to the same extent, skim milk was chosen over BSA because the serum contains polyclonal antibodies against BSA.
6. The primary antibody was incubated for 5 hours at 37° C, chosen over 2 hours, 5 hours and 18 hours of incubation at 37° C. Incubation time of 5 hours was suitable for the assay. Primary antibody-coated plates were treated with 1:10000, 1:15000 and 1:20000 diluted 100 µL of secondary antibody for 45 minutes, 1:20 diluted 100 µL of TMB for 10 minutes and 100 µL of stopping solution to get the result.
7. The indirect assay protocol was defined as follows:
    1. The plate was coated for a day at 4° C with 100 µL of rat C-peptide standards ranging from 0 to 10 ng/mL in 0.05 M phosphate, pH=7.4 with 0.1 % trehalose.
    2. The decanted plate was blocked for an hour with 5 % skim milk in phosphate buffer saline (PBS). The coated plate was washed 5 times with PBST (PBS with 0.1 % tween 20).
    3. The blocked plate was incubated for 5 hours at 37° C with 100 µL of primary antibody (1:1000 dilutions). Washed five times with 300 µL of PBST and tapped to remove any remaining droplets.
    4. 100 µL of 1:20000 diluted secondary antibodies were added immediately and incubated at 37° C for 45 minutes. The plate was aspirated and washed properly.
    5. 100 µL TMB added plates were incubated for 10 minutes at room temperature in dark conditions and the reaction was stopped by 100 µL of 2 M Sulphuric acid. The absorbance of the plate was noted at 450 nm.
8. Standard curve for indirect ELISA was obtained with the equation $y = 0.231x + 0.062$ $R^2=0.974$ with the sensitivity of 0.171 ng/mL. Quality control parameters like recovery were in the range of 93-110 %. The intra-assay variation observed for all the tested samples ranged between 7–10 %. Similarly, the inter-assay

variation was 5–9 % in all the tested samples. Quantitated rat C-peptide concentration from the indirect ELISA method was 0.18 -0.673 ng/mL.

9. The competitive assay involves the competition between the coated rat C-peptide and added rat C-peptide standards. The rat C-peptide concentration of 1, 2 and 3 µg/mL were used to coat the plates and 1 µg/mL is well suitable to coat the plate. The coating was done for a day at RT (25±2º C) or three days at 4° C. Free space in the coated wells was saturated by adding 3 % skim milk solution and the coated plate was stored at -20º C.
10. A mixture of 50 µL each of rat C-peptide standards (0-8 ng/mL) and polyclonal antibody (1:1000) was added. Incubated for 5 hours and addition of secondary antibody, TMB and stopping solution gave the absorbance of 2.84, 2.4, 1.86, 1.36, 1.05, 0.77 and 0.40 for the rat C-peptide concentrations 0, 0.25, 2.5, 1, 2, 4 and 8 ng/mL.
11. The other conditions, like a primary antibody, secondary antibody and substrate incubation, were optimized as 5 hours, 45 minutes and 10 minutes, respectively. The sample volume of 50 µL was suitable for the competitive assay developed. The whole assay system involves washing with PBST (phosphate buffer saline tween) after each step.
12. The competitive assay protocol was optimized as:
    1. The plate was coated with the 100 µL of rat C-peptide standards of 1 ng/mL in 0.05 M phosphate, pH=7.4, with trehalose at RT for a day.
    2. The decanted plate was blocked with 3 % skim milk in phosphate buffer saline (PBS) for an hour and the plate was washed with PBST (PBS with 0.1 % tween 20) 5 times.
    3. The blocked plate was stored at -20 ºC. During the assay process, the pre-coated plate was allowed to react with 50 µL of primary antibody (1:1000 dilution) and 50 µL of rat C-peptide standards (0-8 ng/mL) at 37º C for 5 hours. Rewashed 5 times with 300 µL of PBST and tapped to remove water droplets.
    4. 100 µL of 1:20000 diluted secondary antibodies was added immediately and incubated for 45 minutes at 37º C.
    5. The plate was washed thoroughly before adding 100 µL of TMB. A plate with TMB was incubated for 10 minutes at RT (25±2º C) in a dark place

and the reaction was stopped by the addition of 100 µL of 2 M sulphuric acid.

6. Reading was taken at 450 nm.

13. A standard curve was drawn by taking the rat C-peptide concentration on the x-axis and OD at 450 nm (1/absorbance) on the y-axis. Thus, got standard curve had the equation of $y = 0.2553x + 0.3949$, $R^2 = 0.9901$, the unknown rat serum samples were read using the same equation and the rat C-peptide concentration in the range of 0.17- 0.6 ng/mL was obtained. The sensitivity of the assay was statistically calculated and obtained concentration was 0.15 ng/mL.
14. Correlation studies of the developed indirect ELISA, competitive ELISA and IRMA gave the correlation equation of $y = 1.0107x + 0.0099$ ng/mL $R^2 = 0.9578$, $y = 0.9872x + 0.0024$ ng/mL $R^2 = 0.9983$ and $y = 0.9949x + 0.0068$ ng/mL with $R^2 = 0.9657$, respectively. The obtained mean C-peptide concentration in rat serum was 0.329 ±0.157 ng/mL. The distribution of the rat C-peptide concentration in 30 rat serums ranged between 0.171 to 0.6 ng/mL.

The developed methods have a good sensitivity of 0.24, 0.17 and 0.15 ng/mL for IRMA, indirect ELISA and competitive ELISA, respectively and also covered a good assay range of 0-20 ng/mL. Magnetic particles as a separation system in the rat C-peptide IRMA facilitate the fast separation compared to other available radio-isotopic assays. The minimum sample volume of 100 µL was utilized in all the developed assays to quantify C-peptide in rat serum. The developed methods can be adopted for the C-peptide quantification in anti-diabetic research and minimum use of reagents makes it beneficial for any laboratory.

References:

1. Adienbo OM and Hart VO (2021) Antidiabetic effect of persea americana seed extract is mediated through enhanced insulin secretion, improved beta-cell function, and reduced insulin resistance in diabetic rats. European Journal of Research in Medical Sciences 9 (1): 18-25.
2. Ekins RP (1998) Ligand assays: from electrophoresis to miniaturized microarrays. Clinical Chemistry 44(9): 2015-2030.
3. Eluehike N and Onoagbe IO (2020) Preliminary Nutrient Determination and Regeneration of Pancreatic Islet Cells by Extracts of Spondiasmombin leaves in

Streptozotocin-Induced Diabetic Rats. Avicenna Journal of Medical Biochemistry 8(1): 15-20.

4. Greenwood FC, Hunter WM and Glover JS (1963) The preparation of 131I-labelled human growth hormone of high specific activity. Biochemical Journal 89: 114-123.
5. Horwitz DL, Rubenstein AHMD and Katz AI (1997) Quantitation of Human Pancreatic Beta-cell Function by Immunoassay of C-Peptide in Urine. Diabetes 26(1): 30-35.
6. Horwitz DL, Starr JI, Mako ME, Blackard WG and Rubenstein AH (1975) Proinsulin, insulin, and C-peptide concentrations in human portal and peripheral blood. Journal of Clinical Investigation 55: 1278–1283.
7. Hytest Technotes 2011 Rat C-peptide and proinsulin. Access web: https://hytest.fi/resources/technotes/rat-c-peptide-and-proinsulin-technotes. date: 24/06/2022.
8. Jyotsna N, Singh Y, Chouthkanthiwar V, Paradkar S. and Sivaprasad N (1998) Development of simple immunoradiometric assays using avidin coupled to polystyrene beads as a common solid phase. Conference: International symposium on modern trends in pharmaceuticals for diagnosis, Lisbon (Portugal): 351-358.
9. Kadwad V, Jyotsna N, Sivaprasad N, Sinha P (1996) A method for preparation of magnetizable cellulose and its application in radioimmunoassays for T3 and T4. Journal of Radioanalytical and Nuclear Chemistry 210(1): 27-33.
10. Koivunen ME, and Krogsrud RL (2006) Principles of Immunochemical Techniques Used in Clinical Laboratories. Lab medicine 37: 490-497.
11. Korsgren O, Lundgren T, Felldin M, Foss A, Isaksson B, Permert J, Persson NH, Rafael E, Ryden M, Salmela K and Tibell A (2008). Optimising islet engraftment is critical for successful clinical islet transplantation. Diabetologia 51(2): 227-232.
12. Liu T, Chen M, Ren Z, Hou J, Lin G, and Wu Y (2014) Development of an improved time-resolved fluoroimmunoassay for simultaneous quantification of C-peptide and insulin in human serum. Clinical Biochemistry 47(6): 439-444.
13. Owusu BY, Pflaum H, Garner R, Foulon N, Laha TJ and Hoofnagle AN (2021) Development and validation of a novel LC-MS/MS assay for C-peptide in human serum. Journal of Mass Spectrometry and Advances in the Clinical Lab 19: 1–6.

14. Peterson RG, Shaw WN, Neel MA, Little LA and Eichberg J (1990) Zucker diabetic fatty rat as a model for non-insulin-dependent diabetes mellitus. Ilar Journal 32(3): 16-19.
15. Steiner DF, Clark JL, and Nolan C (1969) Proinsulin and the biosynthesis of insulin. Recent Progress in Hormone Research 25: 207–282.
16. Striano S, Striano P, Manganelli F, Boccella P, Bruno R, Santoro L and Percopo V (2003) Distal hypoglycemic neuropathy. An insulinoma-associated case, misdiagnosed as temporal lobe epilepsy. Neurophysiologie Clinique Clinical Neurophysiology 33(5): 223-227.
17. Sugimoto K and Yagihashi S (1996) Peripheral nerve pathology in rats with streptozotocin-induced insulinoma. Acta neuropathologica 91(6): 616-623.
18. Tager HS and Steiner DF (1972) Primary Structures of the Proinsulin Connecting Peptides of the Rat and the Horse. The journal of biological chemistry 247(24): 7935-7940.
19. Tashtoush BM, Traboulsi A, Dittert LW and Hussain AA (2001) Chloramine-T in Radiolabeling Techniques. Analytical Biochemistry 288(1): 16-21.
20. Thomas A, Yang R, Petring S, Bally L and Thevis M (2020) Simplified quantification of insulin, its synthetic analogs and C-peptide in human plasma by means of LC-HRMS. Drug Test Analysis 12: 382–390.
21. Vrinda C, Paradkar SN, Nagvekar UH, Sivaprasad N, and Pillai MRA (2003) Development of a simple and rapid antibody immobilization procedure for immunoradiometric assay of FSH and HCG. In Nuclear and Radiochemistry Symposium: Proceedings of BRNS-DAE National Symposium on Nuclear and Radiochemistry: 1-11.
22. Wei M, Ong L, Smith MT, Ross FB, Schmid K, Hoey AJ, Burstow D and Brown L (2003) The streptozotocin-diabetic rat as a model of the chronic complications of human diabetes. Heart, Lung and Circulation 12(1): 44-50.
23. Wood WG, Wachter C and Scriba PC (1981) "Experiences using chloramine-t and 1, 3, 4, 6-tetrachloro-3α, 6α-diphenylglycoluril (lodogen®) for radioiodination of materials for radioimmunoassay. Journal of clinical chemistry and clinical biochemistry 19(10):1051-1056.
24. World Health Organization (WHO). Diabetes. Geneva: WHO; published on. 12-Nov-2021. Available from: https://www.who.int/news-room/fact-sheets/detail/diabetes. Accessed Dec 2021.

25. Yue DK, Gibby OM, Luzio SD, Yanaihara N and Hales CN (1979) Indirect two-site immunoradiometric assay of rat and mouse proinsulin. Diabetologia 17(4): 235-242.

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Publications:

1. Manupriya, B R, Shalaka Paradkar, Lathika, Shrikant L Patil, Somashekarappa, H M, and Bhasker K Shenoy. (2018). Optimization of reagent concentration for radioioination of rat C-peptide II in development of radioimmunoassay procedure for rats. Radiation Protection and Environment, 41(1):26-29.

2. Manupriya, B R, Lathika, Somashekarappa, H. M. S.L. Patil, K.B. Shenoy (2020). Study of gamma irradiation effects on the physico-chemical properties of wheat flour (*Triticum aestivum*, L.). Radiation Physics and Chemistry, 172 (108693):1-9.

Communicated research articles:

1. Manupriya B R, Paradkar S. N., Ghodke T. S., Kadwad, V. B., Karunakar N., and Shenoy K. B. (2022). Development of a magnetizable cellulose particles-based immunoradiometric assay for quantification of C-peptide in rat serum. Commumicated to Journal of Radioanalytical and Nuclear Chemistry.

2. Manupriya B R, T. S. Ghodke, Abhiman, P. B. K. Hegde, H. M. Somashekarappa, and K. B. Shenoy, (2022). Developmentand validation of polyclonal antibody based indirect and competitive ELISA for the quantification of rat C-peptide in rat serum for research applications. Commumicated to scandinavian journal of clinical and laboratory investigation.

Presentations:

1. Manupriya B R, T. S. Ghodke, S. N. Paradkar, V. B. Kadwad, & K. B. Shenoy. **Optimization of the immunoradiometric assay (IRMA) for the quantification of C-peptide in rat serum for research applications.** *15th Biennial DAE BRNS Symposium Nuclear and Radiochemistry (NUCAR-2021),* Feb 22-26, 2022, BARC, Mumbai.
2. Manupriya B R, T. S. Ghodke, Karunakara N., Hegde K. & K. B. Shenoy **Preparation and characterization of rat C-peptide-BSA conjugate for production of rat C-peptide polyclonal antibodies for ELISA development. International Conference**. On Advanced Biology 2022 (ICAB 2022)- virtual conference, 23-25 Feb 2022, Inter University Centre for Evolutionary and Integrative Biology (iCEIB) University of Kerala Kariavattom Thiruvananthapuram, Kerala-695581.
3. Manupriya B R, S. N. Paradkar, V. B. Kadwad, Lathika, H. M. Somashekarappa & K. B. Shenoy. "**Identification of capture-detector antibody pair for quantification of rat C-peptide in serum by immunoradiometric method**." "IARPNC – 2020 – virtual conference" 21-23 Jan 2021, BARC, Nuclear Fuel Complex, Hyderabad.
4. Manupriya B R, Lathika, S. L. Patil, H. M. Somashekarappa, & K. B. Shenoy **"Study of Gamma Irradiation Effects on Morphology and crystallinity of wheat flour (*Triticum aestivum*)."** in a "21st National symposium on radiation physics" held on 5-7 Mar 2018 at Raja Ramanna Centre for Advanced Technology (RRCAT), Indore.
5. Manupriya B R, Lathika, V. B. Kadwad, S. N. Paradkar, H. M. Somashekarappa, S. L. Patil, Sachdev, S. S. & K. B. Shenoy "**Optimization of reagent concentration for radioiodination of rat C-peptide II in development of Radioimmunoassay (RIA) procedure for rats"** in a "National conference on Radiation Physics" held on 23-24 Nov 2017 at Bangalore University, Bangalore.
6. Manupriya B R, Lathika, S. L. Patil, H. M. Somashekarappa & K. B. Shenoy **"Optimization of specific activity of Rat C-peptide II for the development of Radioimmunoassay (RIA) procedure"** in a national conference on "Reaching the unreached through science and technology: recent advances in Physical, Chemistry, Mathematical and Biology Sciences for Energy, Health and Environment" held on 8-9 Sep 2017 at Mangalore University, Mangalore.
7. Manupriya B R, Lathika, S. L. Patil, H. M. Somashekarappa & K.B. Shenoy **"Gamma irradiation effects on functional properties of wheat flour."** in the "International conference on radiological safety in work place, nuclear facilities and environment (IARPIC-2016)" held during 22-25 February, 2016 at concention centre, anupuram, Tamilnadu.

8. Manupriya B R, Lathika, S. L. Patil, H. M. Somashekarappa, & K. B. Shenoy **"Assessment of insect infestation in gamma irradiated wheat flour over storage"** in a "International conference on Radiation Research: Impact on Human Health and Environment (ICRR-HEE 2016)" held on 11-13 Feb 2016 at BARC, Mumbai.

9. Manupriya B R, K. B. Shenoy, S. L. Patil & H. M. Somashekarappa, **"Changes in total carbohydrate and total antioxidant activity induced by gamma irradiation of wheat flour"** in the "National conference onrecent advances in animal sciences (NCRAAS)" held during 13-15 February, 2015 at Mangalore University.